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DETERMINATION OF THE ACTIVE COMPONENT(S) OF HEPARIN ASSOCIATED --ETC(U)
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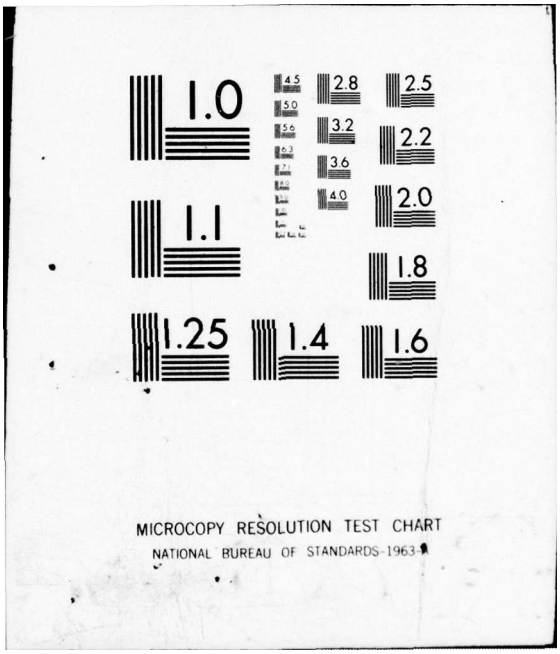
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DETERMINATION OF THE ACTIVE COMPONENT(S) OF HEPARIN ASSOCIATED WITH WOUND HEALING AFTER SEVERE BURN: STRUCTURAL ANALYSIS AND BIOLOGICAL ACTIVITY

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Howard M. Jenkin

Final Report to the Office of Naval Research
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Heparin and casein are two potential burn healing compounds. Their effect on growth of mammalian cells cultivated *in vitro* has been investigated. Heparin appears to have some growth promoting effect on prepuce cells, while it showed little effect on growth of Novikoff hepatoma, monkey kidney and baby hamster kidney cells. Heparin also reversed the inhibitory effect of prednisolone-21-sodium-succinate of growth of prepuce and baby hamster kidney cells.

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20. Partially hydrolyzed casein having an amino N/total N ratio of 0.09 exhibited growth-stimulating activity for prepuce cells which resembles activity of newborn calf serum. This activity was abolished when casein was more fully hydrolyzed with trypsin.

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DETERMINATION OF THE ACTIVE COMPONENT(S) OF HEPARIN ASSOCIATED WITH WOUND HEALING AFTER SEVERE BURN: STRUCTURAL ANALYSIS AND BIOLOGICAL ACTIVITY

Heparin has been shown to accelerate recovery of burn patients and promote wound healing in humans and animals. The mechanism and process of burn and wound healing is still not well understood. Therefore, this study was undertaken to investigate whether heparin can stimulate proliferation of mammalian cells *in vitro* which might be related to the mode of cation of the healing process. Efforts have been made to correlate the physical and chemical properties of heparin to the biological activities associated with cell growth and burn healing.

In addition to heparin, another potential burn healing compound, hydrolyzed casein, was also included in this project. The effect of various forms of hydrolyzed casein on the growth of human skin diploid and other cell types have been investigated.

MAJOR ACCOMPLISHMENTS

1. Facilities and procedures have been established and developed to cultivate many types of mammalian cells *in vitro* for testing effects of a number of drugs on cell growth.

2. Studied the effect of heparin on growth of Novikoff hepatoma, monkey kidney (MK-2), baby hamster kidney (BHK-21) and prepuce cells cultivated *in vitro*.

3. Studied the effects of heparins with different specific activities and heparins from various animal sources and manufacturers on growth of prepuce cells.

4. Studied the combined effect of heparin and prednisolone-21-sodium-succinate (PSS) on growth of BHK-21 and prepuce cells.

5. Studied the effect of various partially hydrolyzed caseins on growth of prepuce cells.

SUMMARY OF THE RESEARCH ACCOMPLISHED:

1. Cell culture techniques:

Procedures have been developed to cultivate *in vitro* Novikoff hepatoma, MK-2, BHK-21, prepuce and many other mammalian cells in either monolayer or shaker cultures. In addition, techniques for establishing a new diploid cell strain, for storing

cells for a long period of time, and recovering cells from storage were also accomplished.

Methods were developed for monitoring the effect of various compounds on cell growth. In monolayer systems, cells at an initial population of 2.0 - 3.0 x 10^5 cells/flask in 25 cm^2 polystyrene cell culture flasks containing 4 ml of medium were incubated at 37°C for several days. The medium was supplemented with/without test compounds. The effect of the compounds was measured by determining viability and cell numbers using a Coulter counter or Biophysics cytograph at varying intervals of time. In a shaker culture system, 30-50 ml of a cell suspension in medium containing an initial population of 3.5 x 10^5 cells/ml in the presence of the test compound was placed in 125 or 250 ml Erlenmeyer screw-cap flasks. The cells were incubated at 37°C in a gyratory shaker, at varying intervals of time, the cell suspension was removed from each flask and cells were enumerated with a Coulter counter or Biophysics cytograph.

2. Effect of heparin on growth of rat Novikoff hepatoma, MK-2, BHK-21 and prepuce cells cultivated in vitro:

Heparin at a concentration of 5-10 µg/ml appears to have growth promoting activity for prepuce cells (30% increase in cell number). Heparin had little effect on the growth of rat Novikoff hepatoma, MK-2 and BHK-21 cells.

3. Effect of heparins with varying specific activities on growth of prepuce cells:

Heparin with varying specific activities and heparins from various manufacturers showed the same magnitude of stimulatory effect on growth of prepuce cells. The purity or the specific activity of heparin had no direct relationship to the stimulatory effect on cell growth. It may also be assumed that impurities or contaminants, if any, in heparin preparations play an insignificant or no role in promoting cell growth in vitro.

4. The combined effect of heparin and PSS on growth of BHK-21 and prepuce cells:

PSS at a concentration of 0.5 or 2 µg/ml inhibited the growth of BHK-21 and prepuce cells. Heparin (2.5 - 20 µg/ml) was added to the growth medium in an attempt to reverse this inhibitory effect of PSS on cell growth. The results demonstrated that heparin reversed the inhibitory effect of PSS on growth of BHK-21 and prepuce cells.

5. Effect of various partially hydrolyzed caseins on growth of prepuce cells:

Partially hydrolyzed casein having an amino N/total N ratio of 0.09 at a concentration of 0.4 - 1.6 mg/ml increased growth of prepuce cells cultivated in vitro 50-100%. When casein was hydrolyzed with trypsin having amino N/total N ratio of 0.36 or 0.70, the growth-stimulating activity for growth of prepuce cells was abolished. Human kidney (SWINK), swine testis (ST-92) and rabbit epithedermal cells (Sflep) were not affected by the partially hydrolyzed casein.

A monitoring system for estimating cell growth activity of prepuce cells by heparin is now available which could be a more specific method for correlating wound healing potential of heparin than the assay for blood coagulation activity. Partially hydrolyzed casein from Kraft Company appears to have greater potential for healing wounds than heparin (see details in accompanying manuscript). Another use for the casein is its property to substitute for serum to cultivate cells in vitro. It is suggested that the growth-stimulating activity of casein is attributed to the casein itself and/or optimal amino acid concentrations and polypeptide fractions.

FINAL CONCLUSION OF THE STUDY

Heparin and casein are two potential burn healing compounds. Their effect on growth of mammalian cells cultivated in vitro has been investigated. Heparin appears to have some growth promoting effects on prepuce cells, while it showed little effect on growth of Novikoff hepatoma, monkey kidney, baby hamster kidney cells. Heparin also exhibited the property to reverse the inhibitory effect of prednisolone-21-sodium-succinate on growth of prepuce and baby hamster kidney cells. Either the direct growth effect of heparin or indirect effect of reversing the inhibition of prednisolone-21-sodium-succinate on growth of prepuce cells can be used as an assay to assess heparin activity. These tests could substitute for the current use of blood coagulation to measure heparin activity. The latter assay does not correlate with wound healing activity.

Partially hydrolyzed casein having an amino N/total N ratio of 0.09 exhibited growth-stimulating activity for prepuce cells which resembles activity of newborn calf serum. This activity was abolished when casein was more fully hydrolyzed with trypsin. Partially hydrolyzed casein (PHCI) can be used to stimulate growth of prepuce cells to perhaps measure wound healing activity in animals and has application for use in substituting for serum in cell-free medium which may be useful to grow many other cell lines and strains where serum is detrimental to the final product.

PUBLICATIONS (attached)

1. T. K. Yang and H. M. Jenkin. The effect of heparin on growth of mammalian cells *in vitro*. Submitted for publication to Proc. Soc. Exp. Biol. Med.

2. H. M. Jenkin, T. K. Yang and L. E. Anderson. Effect of partially hydrolyzed casein on growth of human skin diploid cells *in vitro*. Submitted for publication to Proc. Soc. Exp. Biol. Med.

The Effect of Heparin on Growth of Mammalian Cells *In Vitro*[1]

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Running title: Heparin Effects on Cell Growth

Category: Tissue Culture

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Introduction. Heparin has been used primarily as a therapeutic anticoagulant agent (1), and clinically used to treat inflammatory and allergenic diseases (2). It has also been shown to accelerate recovery of burn patients and promote wound healing in humans and animals (3-5). The mechanism and process of burn and wound healing from these observations is still not well elucidated. It is, therefore, of interest to investigate whether heparin can stimulate proliferation of human skin diploid cells *in vitro* which might be related to the mode of action of the healing process.

The effect of heparin and other acid mucopolysaccharides on the growth of various cell types, mainly malignant cells, has been studied by a number of investigators. The results obtained often have been controversial. Some investigators find inhibitory effects on cell growth (6-8), some stimulatory (8-10), and some report morphological changes (11). Therefore, this study was carried out in an attempt to provide more information about the nature of the effect of heparin on the growth of culture mammalian cells.

Materials and methods. Chemicals. Amino acids, vitamins and newborn calf serum were purchased either from International Scientific Industries Inc., Cary, IL, or Grand Island Biological Co., Grand Island, NY; trypsin (1:250) from Difco Lab, Detroit, MI; prednisolone-21-sodium-succinate (PSS) and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; ethylenediamine-tetraacetic acid (EDTA) from Eastman Kodak Co., Rochester, NY; S-210 medium from Grand Island Biological Co.; Waymouth 652/1 dry powder medium from Schwarz/Mann Inc., Orangeburg, NY; fatty acid-free bovine serum albumin (FAF-BSA) from

Miles Laboratories, Inc., Elkhart, IN; oleic acid from Nu-Chek Prep, Inc., Elysian, MN; various forms of heparin were kindly supplied by Riker Co., Division of 3M Co., St. Paul, MN, Calbiochem, La Jolla, CA, Upjohn Co., Kalamazoo, MI; and highly purified heparin was a gift from Dr. J. A. Cifonelli, University of Chicago, Chicago, IL.

Cell cultures. The sources of the cells and the methods used for cultivation were the same as described previously. Monkey kidney (MK-2) cells were cultivated as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% newborn calf serum (MEM_5) (12). Novikoff hepatoma cells were grown in shaker culture in S-210 medium (13). Human prepuce cells were grown as monolayers in Eagle's MEM medium (14) supplemented with 10% newborn calf serum (MEM_{10}) and baby hamster kidney cells (BHK-21) were grown in shaker culture using a modified Waymouth 752/1 medium (15).

Growth of cells in the presence of heparin. Prepuce cells were grown in Eagle's MEM supplemented with 4% newborn calf serum (MEM_4) or MEM_{10} in the presence of a wide range of heparin. Hanks' balanced salt solution (BSS) was used as a base (16). The cells were used at an initial density of 2.0 - 3.0 x 10^5 cells/flask in a volume of 4 ml and were placed in 25 cm^2 cell culture flasks (Corning Glass Works, Corning, NY). The cells were incubated at 37^o for 6-10 days and were enumerated at varying intervals of time after trypsinization with the aid of a Coulter counter.

Thirty ml of BHK-21 cells were suspended in modified Waymouth 752/1 medium (15) containing 2.5% newborn calf serum and different amounts of heparin. The initial cell population contained 3.5 x 10^5 cells/ml and were incubated at 37^o in a New Brunswick gyratory shaker. At 0, 24, 48, 72 and 96 hr the cells were enumerated with a Coulter counter.

An initial population of 3.0×10^5 MK-2 cells in 4 ml of modified Waymouth 752/1 medium (15) was added to 25 cm^2 flasks. In order to establish monolayers of cells, the medium was supplemented with 1% newborn calf serum. After 24 hr incubation at 37°, the medium was discarded and the cells rinsed with BSS. Fresh modified Waymouth medium without the serum supplement containing different amounts of heparin were added to the cells. The cells were incubated at 37° again and were enumerated at varying intervals of time.

Novikoff hepatoma cells were grown in shaker culture in S-210 medium in the presence of different amounts of heparin. A starting density of 2×10^5 cells/ml was incubated at 37° for 4 days. Cell numbers were determined every 24 hr.

Combined effect of heparin and PSS on the growth of BHK-21 and prepuce cells. Varying amounts of PSS and heparin were added in various combinations to the growth medium to cultivate BHK-21 cells in shaker cultures and prepuce cells in monolayers. The cell growth was measured at varying intervals of time. The procedures were the same as described above for testing the effect of heparin alone.

A minimum of two independent experiments were performed for all studies. Each treatment was carried out in triplicate, and triplicate counts were made on each sample. The results were analyzed for significant differences by using a student's t test.

Results. The effect of Riker's hog mucosal heparin on the growth of prepuce cells cultivated in MEM_{10} is shown in Table I. There were no immediate differences observed in growth between control and heparin-treated cells during the first 3 days after

incubation. On day 5, the cells cultivated in the medium containing 5 and 10 μg/ml of heparin had an increase of 30% and 23%, respectively, in cell numbers over that of the control cells. There was a modest increase in the number of cells grown in medium containing 15 and 20 μg/ml of heparin, whereas the cells treated with 80 μg/ml of heparin had a 21% decrease in cell population when compared to control cells. The population of cells treated with 0, 5, 10 and 20 μg/ml of heparin began to decline after day 5, whereas cells treated with 15 and 80 μg/ml of heparin continued to increase in cell number.

When prepuce cells were cultivated in MEM_4 after initially incubating the cells in MEM_{10} for 24 hr, no differences in the growth between heparin-treated and untreated cells were observed until 8 days after incubation (Table II). On day 8, cells treated with 5 μg/ml of heparin showed a 90% increase in cell numbers over that of the untreated cells. Cells treated with higher concentrations of heparin which were less stimulatory than the cells treated with 5 μg/ml of heparin showed an increase of about 35% in population. On day 10, the cells treated with 5, 10 and 15 μg/ml of heparin all showed about a 30% increase in cell number over the untreated cells. Cells treated with 80 μg/ml of heparin had about the same growth rate as that of the untreated cells.

Various heparins with different anticoagulant activity obtained from Upjohn Co. and Wilson Labs and further purified by J. A. Cifonelli showed similar stimulatory effects on the growth of prepuce cells (Table III). Each of the three heparins at a concentration of 5 μg/ml increased the number of cells about 30-50% from day 5 to 8 after incubation.

Heparins from different sources at a concentration of 5 μg/ml showed similar stimulatory effect on the growth of prepuce cells (Table IV), except there was slightly higher cell population when the cells were grown in the medium containing heparin from porcine intestinal mucosa from Calbiochem.

Heparin showed little or no effect on the growth of MK-2, BHK-21 and Novikoff rat hepatoma cells, when the cells were cultivated in the medium containing several concentrations of newborn calf serum in the presence of a wide range of concentrations of different heparins.

PSS at concentrations of 0.5 μg/ml and 2 μg/ml inhibited the growth of BHK-21 (50%) and prepuce (25%) cells, respectively (Figs. 1 and 2). In an attempt to reverse this inhibitory effect of PSS on cell growth, hog mucosal heparin was added into the culture medium. It was found that cells grown in the medium containing both PSS and heparin had about the same cell numbers as those in mediums containing no PSS (Figs. 1 and 2), showing reversal of heparin on the inhibitory effect by PSS.

Discussion. Previous results about the effect of heparin and other acid mucopolysaccharides on cell growth have been equivocal. Fischer (17) first claimed inhibition of mitosis using heart fibroblasts and concentrations of heparin varying from 20-500 μg/ml. Costachel (7) reported cytotoxic action of heparin at 100-2000 μg/ml using Syrian hamster sarcoma cells. Lippman (8) demonstrated that heparin at 50 μg/ml inhibited growth of mouse "L" cells. King et al. (18) found that heparin at 1-1000 μg/ml showed little effect on cell division of mouse "L" cells grown in suspension cultures.

Our data showed that heparin appears to promote the growth of prepuce cells but did not stimulate growth of BHK-21, MK-2 or Novikoff rat hepatoma cells. Takeuchi (10) noted that acid mucopolysaccharides have some promoting activity on tumor growth. Ozzello et al. (19) reported the growth promoting activity of acid mucopolysaccharides on a strain of human mammary carcinoma cells. They ascribed this action to the negative electric charge and the viscosity of acid mucopolysaccharides.

The controversy about the action of heparin on the cell growth is presumably due in part to different dosages of heparin and the cell types used. Heparin in high concentration can be inhibitory to the growth of cells cultivated in vitro. If the amount of heparin is maintained at a dose that just inhibits coagulation (2 μg/ml), it seems to be relatively non-cytotoxic (11), and perhaps even stimulatory to cell growth. Zakrezewski (20) claimed that the Jensen sarcoma in tissue culture was inhibited by heparin, but emphasized that this drug was much less effective on normal embryonic tissue.

Medium supplemented with low amounts of serum (4%) was used in one set of experiments to hopefully show a growth stimulatory effect when supplemented with heparin. Growth of prepuce cells might then be magnified when cells were subliminally starved (21). From the results shown in Table II, no overall change in the percentage of increase of cells was observed. Medium containing 2% serum was also tested. No stimulatory effect of heparin was noted when prepuce cells were grown in this medium. Takeuchi hypothesized (10) that acid mucopolysaccharides did not serve as a nutritional component for cell growth but protects the cell

surface and promotes the exchange of various metabolites. Our observation indirectly further supports this hypothesis.

Cell populations from monolayer cultures were found to drop in the first 24 hr incubation. This probably is attributed to cell lysis during the trypsinization process. Therefore, the baseline data for all experiments was best interpreted after 24 hr cultivation.

It has been reported that heparin in animal experiments could interact with steroid hormones (2). Our data demonstrated that heparin reversed the inhibitory effect of PSS on the growth of prepuce and BHK-21 cells cultivated in vitro. This test system could be used to indirectly show heparin effects on cell growth when little or no activity was noted by heparin directly. This observation confirms the hypothesis of Dougherty and Dolonitz (2).

A question had been raised whether trace metal contaminants or other unknown contaminants of heparin might be responsible for its activity in aiding burn repair. When crude, commercial grade and highly purified heparins from hog mucosa and/or beef lung sources were tested for promoting cell growth, no differences were found in the activity, which seemed to negate the role of heparin contaminants in the cell culture detection systems used.

Since heparin and heparin-like components are normal constitutents of the blood and cells of higher animals, it is not surprising to find that heparin at a physiological level is harmless and even stimulatory to the cell growth in vitro.

Summary. The effect of heparin on the growth of four cell types cultivated in vitro has been investigated. The results suggest that heparin appears to have some growth promoting

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TABLE I. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM SUPPLEMENTED WITH 10% NEWBORN CALF SERUM

	Heparin (μg/ml)					
Day	0	5	10	15	20	80
1	1.33 ± 0.03[a]	1.27 ± 0.03	1.30 ± 0.00	1.27 ± 0.07	1.17 ± 0.03	1.30 ± 0.12
3	2.97 ± 0.09	3.40 ± 0.21	3.20 ± 0.10	2.60 ± 0.17	2.70 ± 0.27	2.63 ± 0.18
5	4.70 ± 0.10	6.10 ± 0.35[b]	5.77 ± 0.22[b]	5.03 ± 0.07	5.30 ± 0.20	3.73 ± 0.70
7	4.20 ± 0.29	5.67 ± 0.35[b]	5.30 ± 0.47	6.20 ± 0.31[b]	4.53 ± 0.29	4.83 ± 0.46

[a]Average cell number x 10^5/flask (25 cm^2) ± SEM from three flasks each counted in triplicate.

[b]Significantly different from control ($P < 0.05$). These data are typical results from a minimum of three independent experiments.

TABLE II. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM

	Heparin (μg/ml)					
Day	0	5	10	15	20	80
1	1.50 ± 0.07[a]					
2	2.00 ± 0.06	1.93 ± 0.09	1.67 ± 0.07	1.93 ± 0.15	1.93 ± 0.07	2.23 ± 0.07
4	2.77 ± 0.03	2.90 ± 0.10	2.73 ± 0.07	2.73 ± 0.22	2.53 ± 0.07	2.37 ± 0.03
6	2.83 ± 0.03	3.17 ± 0.35	3.17 ± 0.19	3.03 ± 0.18	3.37 ± 0.34	2.60 ± 0.06
8	2.83 ± 0.03	5.40 ± 0.32[b]	3.60 ± 0.36	4.10 ± 0.72	3.83 ± 0.52	3.27 ± 0.43
10	4.87 ± 0.09	6.43 ± 0.23[c]	6.40 ± 0.46[c]	6.30 ± 0.15[b]	5.77 ± 0.09[b]	4.83 ± 0.19

[a]Average cell number x 10^5/flask (25 cm^2) ± SEM from three flasks each counted in triplicate.

[b]Significantly different from control ($P < 0.01$).

[c]Significantly different from control ($P < 0.05$). These data are typical of results from three independent experiments.

TABLE III. EFFECT OF HEPARINS (5 μg/ml) WITH DIFFERENT SPECIFIC ACTIVITIES ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM

	Heparins			
Day	Control	A	B	C
1	1.37 ± 0.09[a]			
3	3.07 ± 0.15	3.27 ± 0.20	3.17 ± 0.13	3.47 ± 0.29
5	3.17 ± 0.09	4.57 ± 0.23[b]	4.33 ± 0.19[b]	4.27 ± 0.22[b]
6	4.03 ± 0.12	6.00 ± 0.40[b]	5.37 ± 0.20[b]	5.63 ± 0.30[b]
8	5.20 ± 0.15	7.10 ± 0.32[b]	6.80 ± 0.46[b]	6.63 ± 0.09[c]

A: Beef lung heparin from Upjohn Co. further purified by gel filtration on Sephadex G-75 by J. A. Cifonelli (specific activity of 144 Iμ/mg).

B: Beef lung heparin from Wilson Labs further purified by J. A. Cifonelli (specific activity of 180 Iμ/mg).

C: Beef lung heparin from Wilson Labs (specific activity of 110 Iμ/mg).

[a]Average cell number x 10^5/flask (25 cm^2) ± SEM from three flasks each counted in triplicate.

[b]Significantly different from control ($P < 0.05$).

[c]Significantly different from control ($P < 0.01$). These data are typical from three independent experiments.

TABLE IV. EFFECT OF VARIOUS HEPARINS (5 μg/ml) ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 10% NEWBORN CALF SERUM

Day	Heparins				
	A	B	C	D	E
1	1.17 ± 0.03[a]	1.17 ± 0.03	1.27 ± 0.03	1.10 ± 0.06	1.23 ± 0.03
3	1.90 ± 0.06	2.30 ± 0.10[b]	2.23 ± 0.09	2.57 ± 0.20[b]	2.90 ± 0.10[c]
5	2.67 ± 0.13	3.13 ± 0.24	3.27 ± 0.35	3.00 ± 0.06	3.77 ± 0.22[b]
7	3.17 ± 0.18	3.73 ± 0.19	5.13 ± 0.39[b]	4.23 ± 0.23[b]	4.10 ± 0.25[b]
9	3.23 ± 0.09	4.10 ± 0.17	4.07 ± 0.03[c]	4.03 ± 0.28	4.40 ± 0.06[c]

A: Control, B: Riker's hog mucosal heparin, C: Riker's beef lung heparin, D: Riker's crude hog mucosal heparin, E: Calbiochem's heparin from hog mucosa.

[a]Average cell number x 10^5/flask ± SEM from three flasks each counted in triplicate.

[b]Significantly different from control ($P < 0.05$).

[c]Significantly different from control ($P < 0.01$). These data are typical from three independent experiments.

Legends to Figures

FIG. 1. Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of BHK-21 cells cultivated in shaker cultures. 30 ml of a cell suspension in modified Waymouth 752/1 medium containing an initial population of 3.5×10^5 cells/ml was placed in 125 ml Erlenmeyer screw-cap flasks. PSS alone or a combination of PSS and heparin was added to the medium. The cells were incubated at 37° and enumerated with a Coulter counter at varying intervals of time. ●——●, 0 µg/ml PSS, heparin; o——o, 0.5 µg/ml PSS; ▲——▲, 0.5 µg/ml PSS + 5 µg/ml heparin; Δ——Δ, 0.5 µg/ml PSS + 10 µg/ml heparin. Vertical bars represent standard error of mean.

FIG. 2. Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of prepuce cells cultivated in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}). Prepuce cells at an initial density of 2.3×10^5 cells/flask in 4 ml of MEM_{10} medium were placed in 25 cm^2 cell culture flasks. PSS alone or a combination of PSS and heparin was added to the medium. The cells were incubated at 37° for 7 days and were enumerated with a Coulter counter at varying intervals of time after trypsinization. ●——●, 0 µg/ml PSS, heparin; o——o, 2 µg/ml PSS; ▲——▲, 2 µg/ml PSS + 5 µg/ml heparin; Δ——Δ, 2 µg/ml PSS + 20 µg/ml heparin. Vertical bars represent standard error of the mean.

FIGURE 1

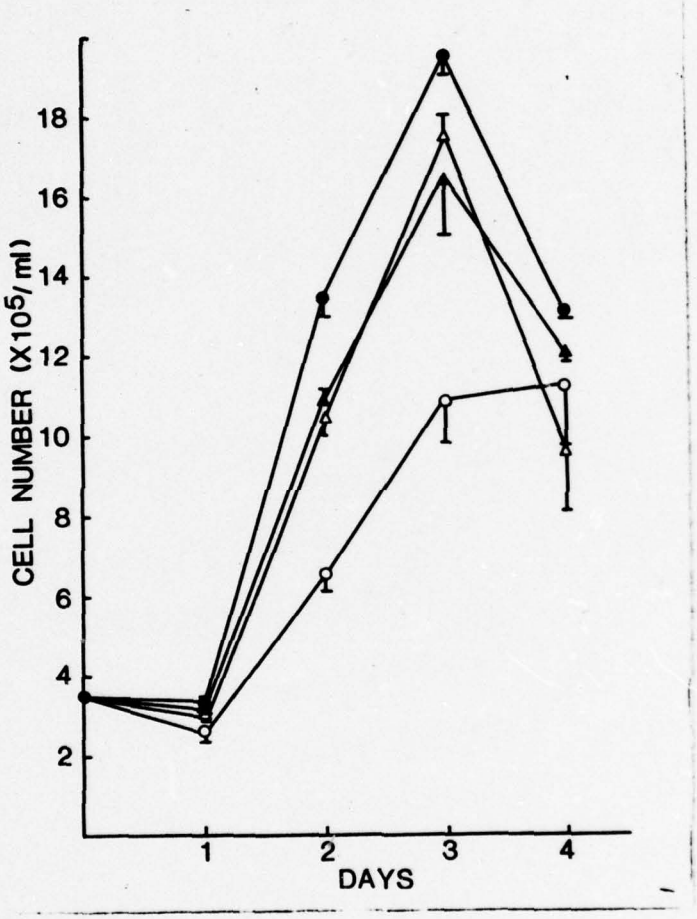
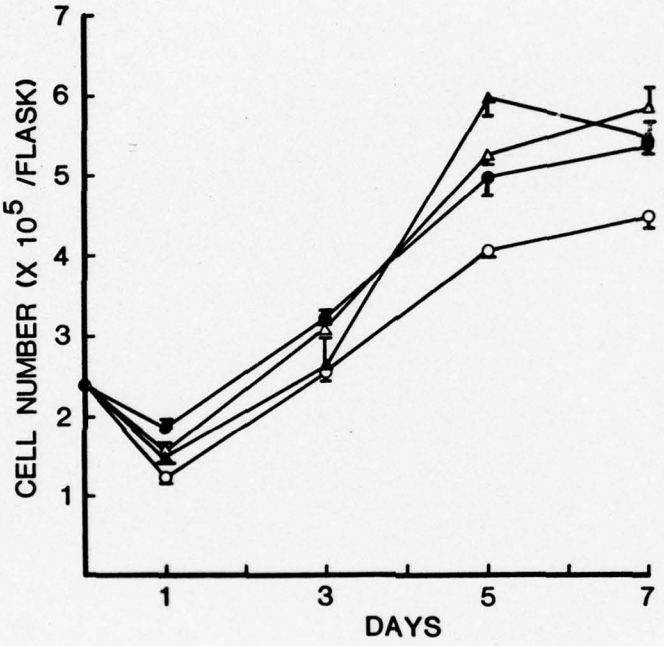


FIGURE 2



Effect of Partially Hydrolyzed Casein on Growth of Human Skin Diploid Cells *In Vitro*[1]

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Casein is a phosphoprotein, precipitated from milk by dilute acid. Partially hydrolyzed casein (PHC) was prepared by hydrolyzing casein with trypsin at 46° for different periods of time to obtain varying amino nitrogen/total nitrogen ratios. This modified casein was thought to have wound healing properties in guinea pigs in preliminary experiments at Kraft Co. (unpublished results). The use of PHC in the local treatment of burns was found to accelerate burn healing (1-3). However, the mechanism of how PHC accelerates burn healing is still not well understood. Curtis, Brewer and Rose (1,2) reasoned that PHC might prevent the loss of tissue protein by the formation of a type of sheltering film in burn treatment. Fleischer (3) speculated that the amino acids of PHC would supply the local nutritional requirements of the damaged cells in burn patients. Serum albumin and polypeptides have been reported to have growth promoting effects on cell growth *in vitro* (4-7).

The experiments in this report were performed in an attempt to investigate whether PHC can stimulate multiplication of human skin diploid cells *in vitro*. This system might be used as a model to study the mode of action of the burn healing process and possibly supplant the use of intact animals for measuring wound repair activity of PHC.

Materials and methods. *Chemicals*. Amino acids, vitamins and newborn calf serum were purchased either from International Scientific Industries, Inc., Cary, IL, or Grand Island Biological Co., Grand Island, NY; trypsin (1:250) from Difco Lab, Detroit, MI; N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; ethylene-diaminetetraacetic acid (EDTA) from Eastman Kodak Co., Rochester, NY and various forms of partially hydrolyzed caseins sterilized with ethylene oxide were generous gifts from Kraft, Inc., Research and Development, Glenview, IL.

Cell culture. Human skin diploid cells (prepuce cells) were established in our laboratory. The cells were prepared from tissue mincings of fresh foreskin of newborn babies according to the procedures of Anderson *et al*. (8) and Sandok *et al*. (9). A number of serial passages of the primary cells were made to establish a diploid cell monolayer culture. The cells were cultivated in 150 cm^2 polystyrene culture flasks (Corning Glass Works, Corning, NY) at 37° using Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) (10). The medium was buffered with 20 mM HEPES (11).

The cell monolayer was subcultured every 4-6 days. The cells were removed from the flask by discarding the growth medium, washing the cells three times with 7 ml of Hanks' balanced salt solution (BSS) (12) without calcium and magnesium salts (GKNP) and finally releasing the cells in 2 ml of 0.05% trypsin/0.05% EDTA in GKNP after 2-3 min of gentle shaking at room temperature. The cells were subcultured at a ratio of 3:1. Fresh medium was added to the cells and incubated at 37°. Cells were stored in fresh medium containing 15% glycerol at -80° in a Revco freezer or liquid nitrogen in vials or ampules containing 5×10^6 cells/ml until used.

Human kidney cells (SWINK) were also screened for growth promoting activity of casein. The cells were obtained from Dr. I. Tribby of the Abbott Laboratories and were cultivated in medium 199 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum. Swine testes cells (ST-92) obtained from Dr. S. McGregor, University of Wisconsin, Madison, and rabbit epidermal cells (SflEp) obtained from Dr. H Fieldsteel, Stanford Research Institute, Menlo Park, CA, were also cultivated in Eagle's MEM (10) supplemented with 10% fetal calf serum.

Growth of prepuce and other cells in the presence of PHC. Prepuce cells were cultivated in Eagle's minimum essential medium containing 0% (MEM_0), 4% (MEM_4) or 10% (MEM_{10}) newborn calf serum in the presence of 0.4 and 1.6 mg/ml PHC. The medium was buffered with 20 mM HEPES and supplemented with 100 µg/ml of streptomycin and 100 units/ml of penicillin. When the effect of PHC was studied on cells cultivated in MEM_0 or MEM_4, the cells were cultivated in MEM_{10} initially for 24 hr. The medium was discarded and cells rinsed with BSS. Fresh medium containing PHC was added to the cells. The experiments were carried out in 25 cm^2 polystyrene cell culture flasks (Corning) at an initial cell density of 2.0 to 3.0 x 10^5 cells/flask containing 4 ml of medium. The cells were incubated at 37° for up to 7-8 days and enumerated at varying intervals of time by removing cells from the flask with trypsin. Cells were counted with the aid of a Coulter counter. A minimum of two independent experiments were performed. Each treatment was carried out in three flasks and triplicate counts were made from each flask.

The SWINK, ST-92 and SflEp cells were tested with PHC in the same manner as prepuce cells.

Results. Prepuce cells after trypsinization lose some viability, therefore to interpret amounts of growth stimulation, the cell population found after 24 hr incubation was used as a baseline control to interpret the data.

The graph presented in Fig. 1 shows the effect of partially hydrolyzed casein I (PHC I) (amino N/total N ratio, 0.09) on the growth of prepuce cells cultivated in MEM_{10} medium. The addition of PHC I to the medium did not significantly change the cell growth compared to the control during the first

3 days of incubation. On day 5, in the presence of 0.4 or 1.6 mg/ml PHC I, increases were observed of about 50% and 113%, respectively, in cell number over the control cells. The number of prepuce cells cultivated in medium supplemented with 1.6 mg/ml PHC I began to decrease after day 5, whereas control cells and to a much lesser degree, cells treated with 0.4 mg/ml PHC I, continued to increase in cell number. Nevertheless, numbers of cells supplemented with 0.4 or 1.6 mg/ml PHC I were still 35% and 64%, respectively, higher than that of control cells on day 7.

The growth stimulating activity of 1.6 mg/ml PHC I on prepuce cells in medium containing 0 or 4% newborn calf serum was similar to that in medium containing 10% newborn calf serum (Fig. 2). Numbers of cells cultivated in MEM_4 supplemented with 1.6 mg/ml PHC I showed increases of 41%, 47% and 31% over that of cells grown in MEM_4 alone on day 3, 6 and 8, respectively.

Cells grown in medium without serum and no PHC I supplement did not multiply over the entire incubation period. Addition of 1.6 mg/ml PHC I to medium containing no serum resulted in 2.5-fold of multiplication after 8 days of incubation.

Figs. 3 and 4 show the effect of partially hydrolyzed casein II and III (PHC II and PHC III) on the growth of prepuce cells cultivated in MEM_{10}. PHC II and PHC III having higher amino N/total N ratios (0.36 and 0.70, respectively) did not appear to exert much effect on the cell growth. On day 3, both caseins slightly stimulated the growth of prepuce cells, whereas on days 5 and 7, the number of cells found in MEM_{10} medium supplemented with PHC II or PHC III were slightly decreased when compared to that of control cells.

The effect of PHC I on growth of human kidney cells (SWINK) is shown in Table I. There is a suggestion that 1.6 mg/ml of PHC I appears to stimulate growth of the cells after 120 to 168 hr of cultivation. Growth-stimulation of SWINK cells was less than prepuce cells in the presence of 1.6 mg PHC.

Swine testis and rabbit epidermal cells did not show any changes in growth in the presence of 0.4 - 1.6 mg/ml of PHC.

Discussion. Polet and Spieker-Polet (4) have reported that serum albumin is essential for growth of activated T and B lymphocytes, and suggested the growth-promoting effect of serum albumin is not due to the fatty acids or hormones bound to serum albumin, but is attributed to the albumin molecule itself or to a factor tightly bound to it. Spieker-Polet and Polet (5) later identified albumin as the serum factor essential for the growth of activated human lymphocytes. Dulak and Temin (6) and Smith and Temin (7) have isolated and purified polypeptides from serum-free medium conditioned by the growth of a line of rat liver cells with growth-stimulating activity for chicken and rat embryo fibroblasts.

The results of the experiments illustrated in Fig. 1 show that PHC I enhanced the growth of prepuce cells cultivated in MEM_{10}. It was found that prepuce cells could multiply to some extent in medium without serum supplement when PHC I was added to the medium (Fig. 2). The growth curve in Fig. 2 also shows that cell populations in medium supplemented with 4% newborn calf serum and PHC I was higher than that of cells grown in medium containing 10% newborn calf serum alone. These observations suggest that PHC I contains growth-stimulating activity for growth of prepuce cells which resembles activity of newborn calf serum.

Spieker-Polet and Polet (5) have shown that the growth-promoting activity of albumin for growth of activated lymphocytes was completely abolished by pepsin digestion. Our results support this finding. When casein was hydrolyzed for extended periods of time with trypsin, a product having amino N/total N ratio of 0.36 or 0.70 was obtained, destroying the growth-stimulating activity for prepuce cells (Figs. 3 and 4). There is a suggestion that the growth-stimulating activity of casein is attributed to the casein itself as well as presence of optimum amino acid concentration and/or polypeptide fractions. Comparison of amino acid and polypeptide analyses of trypsinized casein fractions could further quantitate the optimal amounts of these materials for cell growth.

PHC I could be used to replace serum in cell culture studies. Only prepuce cells, of the four cells screened, appear to show consistent patterns of increase of growth in the presence of PHC I. The specific requirements of components of the medium for optimal growth of human kidney, swine testis and rabbit epidermal cells in the presence of PHC I requires further investigation and was not within the scope of these studies. It appears that cells that grow slowly *in vitro* may be more easily stimulated by PHC I than fast growing cells.

An assay for PHC I in prepuce cell cultures could be eventually used to standardize doses of sterile casein for burn or other wound healing treatments, substituting in part animal efficacy tests.

Summary. The effect of various partially hydrolyzed caseins (PHC) on the growth of human skin diploid (prepuce) cells cultivated *in vitro* has been

investigated. The results indicate that PHC I (amino N/total N ratio of 0.09) contains growth-stimulating activity for prepuce cells in the absence and presence of serum. This activity of PHC I was abolished when casein was further hydrolyzed with trypsin (amino N/total N ratio of 0.36 or 0.70). It is suggested that the growth-stimulating activity of casein is attributed to the casein itself or the presence of optimal amounts of amino acids and/or polypeptide fractions. Human kidney (SWINK), swine testis (ST-92) and rabbit epidermal cells (SflEp) showed little or no growth stimulation in any of the concentrations of PHC I tested. A cell culture system to assay PHC I activity is now available which could eventually short-circuit the routine use of animal assays to test burn or other wound healing cell activity. PHC I appears to substitute for serum in growth of prepuce cells *in vitro* so that another serum-free medium is now available for certain types of experiments.

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The opinions and statements contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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TABLE I. EFFECT OF VARYING AMOUNTS OF CASEIN HYDROLYSATE I (PARTIALLY HYDROLYZED) ON HUMAN KIDNEY CELLS (SWINK).

Casein supplement (%)	Hours incubation - 37°				
	0	24	72	120	168
0.0	3.7[a]	3.1	8.0	6.9	7.2
0.4	5.6	4.1	7.7	7.2	9.3
0.8	3.3	2.2	7.9	8.5	10.0
1.6	4.0	3.3	8.7	9.3	12.0
3.2	4.5	2.5	8.2	8.1	11.0

[a]Cell count/flask x 10^5. Cells were cultivated in medium 199 (Grand Island Biologicals) supplemented with 10% fetal calf serum. The results are typical of two independent experiments. Three samples from each of three flasks were counted in triplicate. Counts were performed on a Coulter counter or Biophysics cytograph.

FIG. 1. Effect of partially hydrolyzed casein I (PHC I, amino N/total N ratio of 0.09)in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) on growth of prepuce cells. An initial inoculum of 2.8×10^5 prepuce cells in 4 ml of MEM_{10} supplemented with PHC I was placed in 25 cm^2 polystyrene cell culture flasks and incubated at 37° for 7 days. Cells were enumerated with a Coulter counter at varying intervals of time after incubation. Each point represents the mean ± SEM of triplicate counts from three flasks. The results are typical of six independent experiments. ●——●, MEM_{10}; o——o, 0.4 mg/ml PHC I in MEM_{10}; ▲——▲, 1.6 mg/ml PHC I in MEM_{10}.

FIG. 2. Effect of partially hydrolyzed casein I (PHC I) in Eagle's minimum essential medium supplemented with 0% (MEM_0) or 4% (MEM_4) newborn calf serum on growth of prepuce cells. An initial inoculum of 2.5×10^5 cells in 4 ml of MEM_{10} was placed in 25 cm^2 polystyrene cell culture flasks. After 24 hr incubation at 37° the medium was discarded and the cells rinsed with Hanks' balanced solution (BSS). MEM_0 or MEM_4 supplemented with or without PHC I was added to the cells and incubated at 37° for 7 more days. Unsupplemented MEM_0 and MEM_4 media were used as controls. Each point represents the mean ± SEM of triplicate cell counts from three flasks. The results are typical of at least two independent experiments. ■——■, cells grown in MEM_{10}; ●——●, cells grown in MEM_0; o——o, cells grown in MEM_0 + 1.6 mg/ml PHC I; ▲——▲, cells grown in MEM_4; Δ——Δ, cells grown in MEM_4 + 1.6 mg/ml PHC I.

FIG. 3. Effect of partially hydrolyzed casein II (PHC II , amino N/ total N ratio of 0.36) in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) on growth of prepuce cells. An initial inoculum of 2.3×10^5 prepuce cells in 4 ml of MEM_{10} containing PHC II was placed in 25 cm^2 polystyrene cell culture flasks and incubated at 37° for 7 days. Each point represents the mean $\pm$ SEM of triplicate counts from three flasks. The results are typical of two independent experiments. ●——●, control; o——o, 0.4 mg/ml PHC II; ▲——▲, 1.6 mg/ml PHC II.

FIG. 4. Effect of partially hydrolyzed casein III (PHC III , amino N/ total N ratio of 0.70) in Eagle's minimum essential medium supplemented with 10% newborn calf serum (MEM_{10}) on growth of prepuce cells. An initial inoculum of 2.5×10^5 prepuce cells in 4 ml of MEM_{10} containing PHC III was placed in 25 cm^2 polystyrene cell culture flasks and incubated at 37° for 7 days. Each point represents the mean $\pm$ SEM of triplicate counts from three flasks. The results are typical of two independent experiments. ●——●, control; o——o, 0.4 mg/ml PHC III; ▲——▲, 1.6 mg/ml PHC III.

FIGURE 1

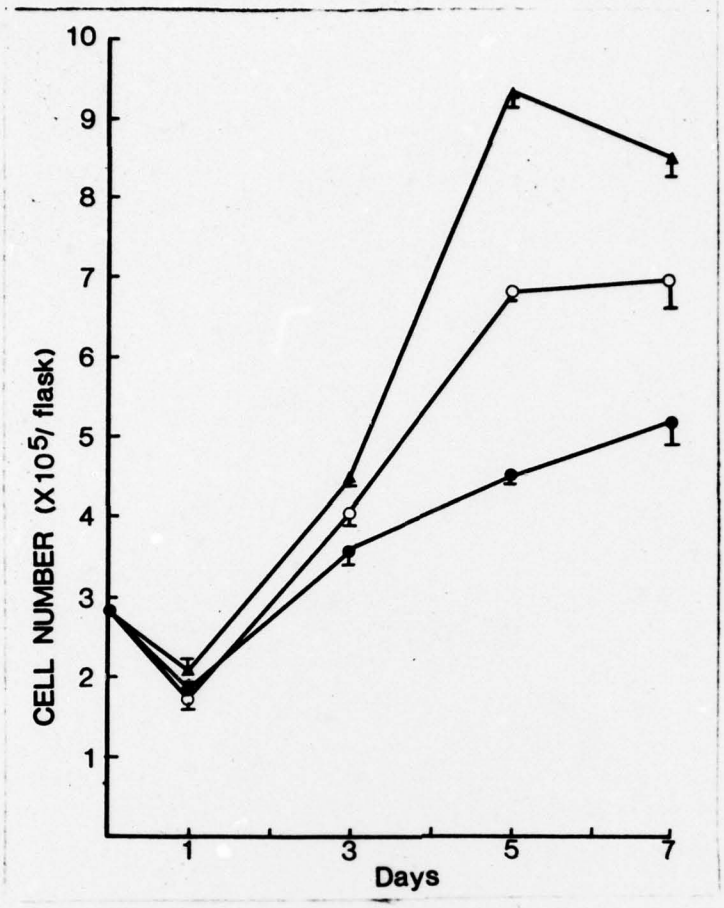

FIGURE 2

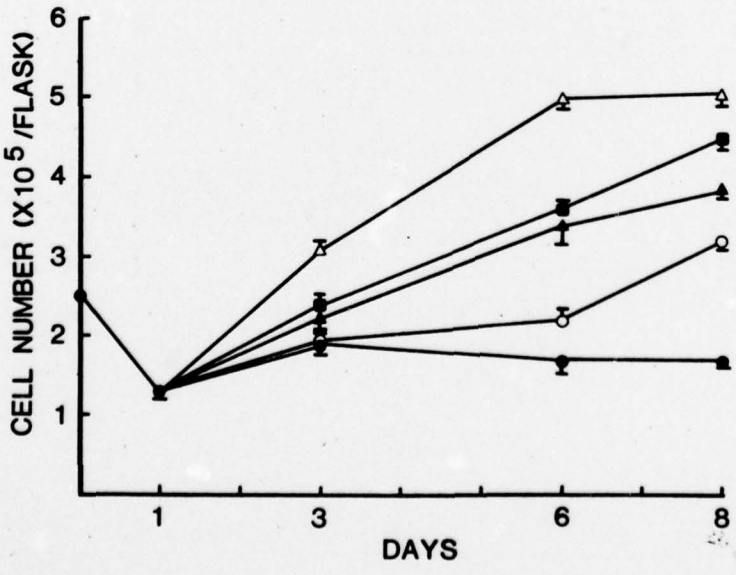

FIGURE 3

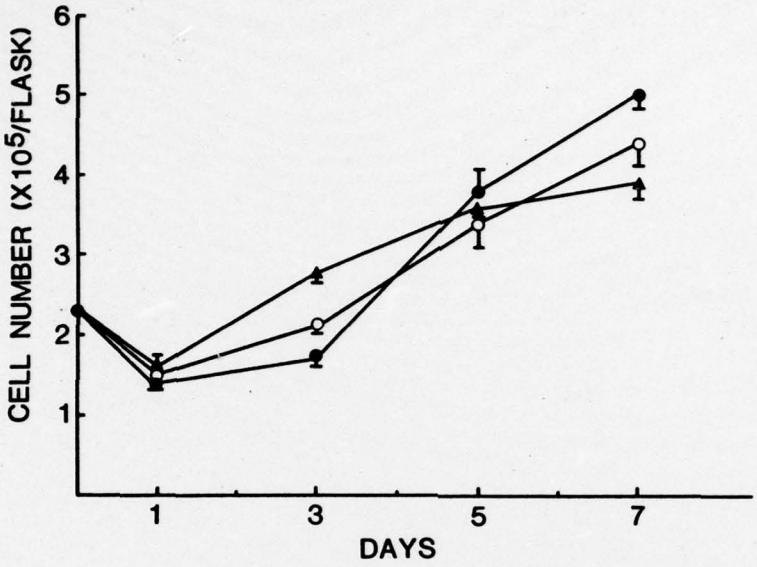

FIGURE 4